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(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: WOLDIKE, Helle, Fabricius [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). KJELDSEN, Thomas, Børglum [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK).

(74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).

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(54) **Title:** A PROCESS FOR PRODUCING TRYPSIN (TRYPSINOGEN)

(57) **Abstract**

Trypsin (trypsinogen) may be produced in a filamentous fungus by transforming a filamentous fungus with a vector comprising a DNA sequence encoding protrypsin or a derivative thereof N-terminally fused to a DNA sequence encoding a signal peptide, culturing the transformed filamentous fungus in a suitable culture medium to produce trypsinogen and recovering trypsinogen and/or trypsin from the medium.

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Title:A PROCESS FOR PRODUCING TRYPSIN (TRYPSINOGEN)

FIELD OF THE INVENTION

The present invention relates to a process for the production of trypsins in filamentous fungi and to DNA sequences to be used in such processes.

BACKGROUND OF THE INVENTION

In recent years, procedures have been developed for the transformation of filamentous fungi, including Aspergillus niger, Aspergillus oryzae, and Aspergillus nidulans. US 4,885,249 (Allelix) describes a general process for the transformation of A. niger, exemplified by the introduction of plasmids carrying genes encoding selectable markers.

This method is generally used for the expression and production of proteins originating from other microbial sources, but mammalian proteins have also been produced in such systems.

However, it has been experienced that the expression of trypsins, especially mammalian trypsins only is accomplished to extremely low levels.

SUMMARY OF THE INVENTION

It has surprisingly been found that when the genes encoding selected trypsinogens (protrypsins) are expressed in Aspergillus sp. the levels of trypsin secreted are increased several fold compared to those apparent from other microbial systems.

Accordingly, the present invention relates to a process for the production of trypsins (trypsinogens) or derivatives thereof in filamentous fungi, the process comprising

(a) transforming a filamentous fungus host organism with a recombinant DNA vector which comprises a DNA sequence encoding trypsinogen (protrypsin) or a derivative thereof N-terminally fused to a DNA sequence encoding a signal peptide that may be the native sequence or another signal sequence derived from a fungus, such as the Aspergillus oryzae TAKA amylase gene or a derivative of such a signal peptide,

(b) culturing the transformed filamentous fungus host organism in a suitable culture medium under conditions conducive to the expression of trypsinogen (protrypsin) and secretion of the trypsinogen and trypsin to the medium, and

(c) recovering the protrypsin or trypsin or derivative thereof from the medium.

In the present context, the term "derivative" is intended to indicate a polypeptide which is derived from the native trypsin or signal peptide (as the case may be) by suitably modifying the DNA sequence coding for the native trypsin/signal peptide, resulting in the addition of one or more amino acid at either or both the C- or N-terminal end, substitution of one or more amino acids at one or a number of different sites in the native amino acid sequence, deletion of one or more amino acids at either or both ends of the native amino acid sequence or at one or more sites within the native sequence, or insertion of one or more amino acids at one or more sites in the native amino acid sequence. Such modifications of the DNA sequence may be done by methods well known in the art.

The term "filamentous fungus" is intended to include the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and

fungi imperfecti, including Hyphomycetes such as the genera Aspergillus, Penicillium, Trichoderma, Fusarium and Humicola.

The presence of the signal sequence serves to direct the expressed trypsinogen or derivative thereof effectively into the secretory pathway of the host cell so that trypsinogen or trypsin may readily be isolated from the culture medium (at least some of the product recovered will be mature trypsin as the trypsinogen secreted from the cells is either subjected to automaturation or maturation by proteases produced by the host cell).

In the present invention the signal sequence does not seem to be critical, and a number have been tested, such as the TAKA-amylase (ref. EP 0 238 023), the PTRYP-trypsin, and the human HTRYPI-trypsin and HTRYPII signal sequences (Okayama et al., Methods in Enzymology **154**, 3-28 (1987), Emi et al., Gene **41**, 305-310, (1986)).

The trypsin (trypsinogen) to be produced by the process of the invention is trypsin of any origin, especially mammalian trypsin, such as porcine, bovine, and human trypsin.

The invention furthermore comprises certain DNA sequences coding for porcine trypsin (trypsinogen) and alleles thereof capable of expressing trypsins having retained their biological activity.

Furthermore the invention relates to vectors comprising said DNA sequence and hosts transformed therewith.

BRIEF DESCRIPTION OF THE TABLES AND DRAWING

The invention is described in further detail in the following parts of the specification with reference to the Examples and the drawing, wherein

Fig.1 shows the steps involved in the construction of pHW470,

Fig. 2 shows the steps involved in the construction of pHW473.

and

Fig. 3 shows the steps involved in the construction of pHW874,

DETAILED DESCRIPTION OF THE INVENTION

As indicated the present invention in its first aspect relates to a process for the production of trypsins (trypsinogens) or derivatives thereof in filamentous fungi, the process comprising

(a) transforming a filamentous fungus host organism with a recombinant DNA vector which comprises a DNA sequence encoding trypsinogen or a derivative thereof N-terminally fused to a DNA sequence encoding a signal peptide that may be the native sequence or another signal sequence derived from a fungus, such as the Aspergillus oryzae TAKA amylase gene or a derivative of such a signal peptide,

(b) culturing the transformed filamentous fungus host organism in a suitable culture medium under conditions conducive to the expression of protrypsin and secretion thereof to the medium, and

(c) recovering the trypsinogen or trypsin or derivative thereof from the medium.

The vector may further comprise DNA sequences encoding functions facilitating gene expression, typically a promoter, transcription initiation sites, and transcription termination and polyadenylation functions.

The promoter which may be preceded by upstream activating sequences and enhancer sequences as known in the art may be any DNA sequence exhibiting a strong transcriptional activity in Aspergillus sp., such as A. oryzae and A. niger, and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α-amylase, A. niger acid stable α-amylase, A. niger glucoamylase, Rhizomucor miehei lipase, or A. oryzae alkaline protease. Examples of promoters from genes encoding glycolytic enzymes are the A. oryzae triose phosphate isomerase, ADH and PGK promoters.

The filamentous fungus used as the host organism is preferably selected from an Aspergillus sp. such as A. niger A. awamori or A. oryzae.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform the host organism may suitably be adapted from the methods of transforming A. nidulans described in, for instance, Yelton et al., Proc. Natl. Acad. Sci. USA 81, 1984, pp. 1470-1474, or EP 0 215 594, from the methods of transforming A. niger described in, for instance Buxton et al., Gene 37, 1985, pp. 207-215 or US 4,885,249, or from the methods of transforming A. oryzae described in EP 238023. In the process of the present invention, A. oryzae or A. niger may be transformed with a vector system comprising a

DNA sequence coding for a selection marker which is capable of being incorporated in the genome of the host organism on transformation, but which is either not expressed by the host before transformation or not expressed in sufficient amounts to permit growth under selective conditions. Transformants can then be selected and isolated from non-transformants on the basis of the incorporated selection marker.

Suitable selection markers may be derived from the A. nidulans or A. niger argB gene, the A. nidulans trpC gene, the A. nidulans amdS gene, the Neurospora crassa pyr4 or DHFR genes, or the A. niger or A. oryzae niaD gene.

Preferred selection markers for use in the present invention are derived from the A. nidulans or A. niger amdS or argB genes. If argB is chosen as the selection marker, an ArgB$^-$ mutant strain (which does not express the ArgB gene) must be used as the host organism. On the other hand, the amdS gene may be used as the selection marker in wild-type A. oryzae or A. niger strains which do not express this gene in sufficient amounts to permit growth under selective conditions.

The signal sequence may be chosen from signal sequences derived from the trypsinogen gene itself, or from a gene encoding e.g. A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α-amylase, A. niger acid stable α-amylase, A. niger glucoamylase, Rhizomucor miehei lipase, or A. oryzae alkaline protease. Examples of genes encoding glycolytic enzymes are the A. oryzae triose phosphate isomerase, ADH and PGK. Combinations and/or variants of such signal sequences may also be used.

The gene coding for trypsinogen fused to the signal sequence as well as to promoter and terminator sequences may be inserted in a vector containing the selection marker, or it may be inserted in a separate vector for introduction into the host cell. The vector or vectors may be linear or closed circular molecules.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing filamentous fungi. The transformants are usually stable and may be cultured in the absence of selection pressure. However, if the transformants are found to be unstable, the selection marker introduced into the cells may be used for selection.

The trypsinogen or trypsin produced by the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

The invention furthermore comprises certain DNA sequences coding for porcine trypsin (trypsinogen) and alleles thereof capable of expressing trypsins having retained their biological activity.

The invention relates in a further aspect to vectors comprising said DNA sequences.

The invention also encompasses hosts transformed with such vectors. The hosts may be of animal or microbial origin, such as mammalian cell lines, bacteria, yeasts or fungi, especially filamentous fungi.

Finally the invention relates to a method of recombinantly producing porcine trypsin, the process comprising

(a) transforming a host with a recombinant DNA vector which comprises a DNA sequence encoding porcine trypsinogen or a derivative thereof N-terminally fused to a DNA sequence encoding a signal peptide that may be the native sequence or another signal sequence or a derivative of such a signal peptide,

(b) culturing the transformed host in a suitable culture medium under conditions conducive to the expression of porcine trypsinogen and secretion thereof to the medium, and

(c) recovering the porcine trypsinogen or trypsin or derivative thereof from the medium.

The invention is further illustrated in the following examples which are not in any way to be construed as limiting to the scope of the invention as claimed.

MATERIALS AND METHODS

EXAMPLES

Example 1.

Cloning of human trypsinogen I and II cDNA.

From a human pancreatic cDNA library constructed according to Okayama et al., Methods in Enzymology 154, 3-28 (1987), we isolated cDNA clones encoding the two major human trypsinogen isozymes, TRYI and TRYII. The sequences of Emi et al., Gene 41, 305-310, (1986), were used to select probes for isolation :

NOR 948 : 5' GCCCCCAACGATCTTGTCATCATCATC 3' SEQ ID NO: 3
NOR 949 : 5' GTTCAGAGTCTTCCTGTCGTATTGGGG 3' SEQ ID NO: 4

NOR 948 is common to TRYI and TRYII, NOR 949 is specific for TRYII. Full length clones were isolated having sequences in accordance with the ones published by Emi et al., Gene 41, 305-310 (1986). The plasmids were designated pHW468 for TRYI and pHW469 for TRYII.

Example 2.

Cloning of porcine trypsinogen cDNA.

mRNA was purified from porcine pancreas using standard methods (Maniatis 1982). cDNA was prepared from the mRNA, purified and inserted into λgt11 using the cDNA cloning system-λgt11 from Amersham, UK. Preparation of phage, plating cells, infection with λgt11, amplification and screening was performed according to the manufacturers introductions and standard techniques (Maniatis 1982). The oligonucleotide NOR 948, as described above, was used for screening of plaques.

Positive plaques were isolated and amplified. The isolated λgt11 DNA was subjected to digestion with EcoR1 and the inserted cDNA was cloned into EcoR1 cleaved pBluescript SK (Stratagene) using ampicillin selection of E. coli JM101 transformants. The selected plasmid was shown by DNA sequencing analysis (Sequenase, U.S. Biochemical Corp.) to contain a cDNA sequence compatible with the known porcine trypsin amino acid sequence (Hermodson et al., Biochemistry 12, 3146-3153 (1973)). The almost complete sequence lacking the very N-terminal end of the signal peptide of porcine pre-pro-trypsin was obtained from 2 EcoR1 fragments of 130 bp covering the N-terminal and 740 bp covering the C-terminal. The resulting plasmid was designated p185, the sequence of which is shown in SEQ ID NO: 1.

Example 3

Expression of human trypsinogen I and II in A. oryzae.

Vectors for expression of human trypsinogen I and II in Aspergillus were constructed as outlined in Fig. 1 and Fig. 2. The BamH1-PvuII linker :

NOR 971 : 5' GATCCACCATGAATCCACTCCTGATCCTTACCTTTGTGGCAG 3'
NOR 972 : 3' GTGGTACTTAGGTGAGGACTAGGAATGGAAACACCGTC 5'
SEQ ID NO: 5

connects the cDNA to the BamH1 site in the fungal expression vector p777 described in EP 0 238 023. The common linker covers the first 11 amino acids of the signal sequence of TRYI, differing only in position 3 from TRYII, which has a leucine instead of proline in its native sequence. The remaining part of the sequence is native to both species.

The trypsinogen expression vectors pHW470 and pHW473 were transformed into A. oryzae IFO 4177, or a protease deficient derivative thereof, A560-T40, using the procedure described in EP 238023. Selection on acetamide was performed by co-transformation with pToC 186 as described in WO 93/00426.

Transformants were grown in YPD medium (Sherman et al.,Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) for 3-4 days and analysed for new protein species in the supernatant by SDS-PAGE and Western blot, using polyclonal antibody raised against porcine trypsin, which did not appear to detect the human trypsin species. However, activity assays using L-BAPNA (L-Benzoyl-arginyl-p-nitro anilide) as substrate demonstrated convincingly the expression of TRYI and TRYII from A. oryzae. Also, both species were purified from A. oryzae supernatants.

Example 4

Expression of porcine trypsin in A. oryzae

A vector for expression of porcine trypsinogen in Aspergillus was constructed as outlined in Fig. 3. To connect the first 18 amino acids of the TAKA amylase signal to the last 4 amino acids of the porcine trypsin signal, we used a Ban1-EcoR1 linker :

226/223:

5' GCACCGGCCGCGGTGGCCTTCCCGACCGACGATGACGACAAGATCGTCGGCGGG

3' GCCGGCGCCACCGGAAGGGCTGGCTGCTACTGCTGTTCTAGCAGCCGCCC

225/224:

TACACGTGTGCAGCGAACTCGATCCCTTACCAGGTCTCGCTG 3' 96 b

ATGTGCACACGTCGCTTGAGCTAGGGAATGGTCCAGAGCGACTTAA 5' 99 b

SEQ ID NO: 6

This fusion also has a part of the TAKA amylase promoter and the N-terminal end of the trypsin gene. The C-terminal region of the trypsin gene is joined to this in Sub2, keeping track of the orientations. The final expression vector, pHW874, has TAKA amylase promoter and AMG terminator as functional elements. These elements were derived from pHD414, which is described in EP 0 505 311.

The porcine trypsin expression vector pHW874 was transformed into A. oryzae as described in Example 3. Transformants were grown in YPD medium and analysed by SDS-PAGE-Western and by cleavage of L-BAPNA, as described in Example 3. In this case distinct bands of the expected size for porcine trypsinogen and mature trypsin were seen on Western blots, corresponding to activity measurements with L-BAPNA.

REFERENCES CITED IN THE SPECIFICATION

US 4,885,249 (Allelix)

Okayama et al., Methods in Enzymology **154**, 3-28 (1987)

Emi et al., Gene **41**, 305-310, (1986)

Yelton et al., Proc. Natl. Acad. Sci. USA 81, 1984, pp. 1470-1474

EP 0 215 594

Buxton et al., Gene 37, 1985, pp. 207-215

US 4,885,249

EP 0 238 023

Hermodson et al., Biochemistry 12, 3146-3153 (1973)

WO 93/00426.

Sherman et al.,Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981

EP 0 505 311

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: DENMARK
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 44448888
(H) TELEFAX: +45 44493256

(ii) TITLE OF INVENTION: Process for the production of trypsin (trypsinogen)

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 897 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Sus scrofa
(F) TISSUE TYPE: Pancreas

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:4..744

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
GGA ATT CCG AAC ACC TTT GTC TTG CTT GCG CTC CTG GGA GCT GCT GTT          48
    Ile Pro Asn Thr Phe Val Leu Leu Ala Leu Leu Gly Ala Ala Val
      1               5                  10                  15

GCT TTC CCC ACG GAT GAT GAT GAC AAG ATC GTC GGG GGT TAC ACC TGT          96
Ala Phe Pro Thr Asp Asp Asp Asp Lys Ile Val Gly Gly Tyr Thr Cys
                  20                  25                  30

GCA GCA AAT TCC ATT CCC TAC CAG GTG TCC CTG AAT TCT GGC TCC CAC         144
Ala Ala Asn Ser Ile Pro Tyr Gln Val Ser Leu Asn Ser Gly Ser His
              35                  40                  45

TTC TGT GGT GGG TCC CTC ATC AAC AGC CAG TGG GTG GTG TCT GCT GCT         192
Phe Cys Gly Gly Ser Leu Ile Asn Ser Gln Trp Val Val Ser Ala Ala
          50                  55                  60
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CAC TGC TAC AAG TCC CGA ATC CAG GTG CGT CTG GGA GAA CAC AAC ATC          240
His Cys Tyr Lys Ser Arg Ile Gln Val Arg Leu Gly Glu His Asn Ile
 65                  70                  75

GAC GTC CTT GAG GGC AAT GAG CAA TTC ATC AAT GCC GCC AAG ATC ATC          288
Asp Val Leu Glu Gly Asn Glu Gln Phe Ile Asn Ala Ala Lys Ile Ile
 80                  85                  90                  95

ACC CAC CCC AAT TTC AAT GGA AAT ACC TTA GAT AAC GAC ATC ATG CTG          336
Thr His Pro Asn Phe Asn Gly Asn Thr Leu Asp Asn Asp Ile Met Leu
                100                 105                 110

ATT AAA CTG AGC TCA CCT GCC ACT CTC AAC AGT CGA GTA GCA ACT GTC          384
Ile Lys Leu Ser Ser Pro Ala Thr Leu Asn Ser Arg Val Ala Thr Val
            115                 120                 125

TCA CTG CCA AGA TCT TGT GCA GCT GCT GGT ACC GAG TGT CTC ATC TCT          432
Ser Leu Pro Arg Ser Cys Ala Ala Ala Gly Thr Glu Cys Leu Ile Ser
        130                 135                 140

GGC TGG GGC AAC ACC AAA AGC AGT GGC TCC AGC TAC CCT TCG CTC CTG          480
Gly Trp Gly Asn Thr Lys Ser Ser Gly Ser Ser Tyr Pro Ser Leu Leu
    145                 150                 155

CAA TGC CTG AAG GCC CCC GTC CTA AGT GAC AGT TCT TGC AAG AGT TCC          528
Gln Cys Leu Lys Ala Pro Val Leu Ser Asp Ser Ser Cys Lys Ser Ser
160                 165                 170                 175

TAC CCA GGC CAG ATC ACC GGA AAC ATG ATC TGT GTC GGC TTC CTG GAG          576
Tyr Pro Gly Gln Ile Thr Gly Asn Met Ile Cys Val Gly Phe Leu Glu
                180                 185                 190

GGT GGT AAG GAT TCT TGC CAG GGA GAC TCT GGT GGC CCC GTG GTC TGC          624
Gly Gly Lys Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro Val Val Cys
            195                 200                 205

AAT GGA CAG CTC CAG GGT ATT GTC TCT TGG GGC TAT GGC TGC GCC CAG          672
Asn Gly Gln Leu Gln Gly Ile Val Ser Trp Gly Tyr Gly Cys Ala Gln
        210                 215                 220

AAA AAC AAG CCT GGG GTC TAC ACC AAG GTC TGC AAC TAT GTG AAC TGG          720
Lys Asn Lys Pro Gly Val Tyr Thr Lys Val Cys Asn Tyr Val Asn Trp
    225                 230                 235

ATT CAG CAG ACC ATC GCT GCC AAC TAAAGAATTT CATTTCTTCA TGACTCTTCC         774
Ile Gln Gln Thr Ile Ala Ala Asn
240                 245

CTTTAGTCAT CTTCACCTTC CTCCCATCCT GCGAACAGCA TCTAAATAAA AACATTTTGA        834

CCTGTACCAG CATCTAAATA AAAACATTTT GAGCTGTACC CAAAAAAAAA AAAAAGGAAT        894

TCC                                                                      897
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 247 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ile Pro Asn Thr Phe Val Leu Leu Ala Leu Leu Gly Ala Ala Val Ala
1 5 10 15

Phe Pro Thr Asp Asp Asp Asp Lys Ile Val Gly Gly Tyr Thr Cys Ala
20 25 30

Ala Asn Ser Ile Pro Tyr Gln Val Ser Leu Asn Ser Gly Ser His Phe
35 40 45

Cys Gly Gly Ser Leu Ile Asn Ser Gln Trp Val Val Ser Ala Ala His
50 55 60

Cys Tyr Lys Ser Arg Ile Gln Val Arg Leu Gly Glu His Asn Ile Asp
65 70 75 80

Val Leu Glu Gly Asn Glu Gln Phe Ile Asn Ala Ala Lys Ile Ile Thr
85 90 95

His Pro Asn Phe Asn Gly Asn Thr Leu Asp Asn Asp Ile Met Leu Ile
100 105 110

Lys Leu Ser Ser Pro Ala Thr Leu Asn Ser Arg Val Ala Thr Val Ser
115 120 125

Leu Pro Arg Ser Cys Ala Ala Ala Gly Thr Glu Cys Leu Ile Ser Gly
130 135 140

Trp Gly Asn Thr Lys Ser Ser Gly Ser Ser Tyr Pro Ser Leu Leu Gln
145 150 155 160

Cys Leu Lys Ala Pro Val Leu Ser Asp Ser Ser Cys Lys Ser Ser Tyr
165 170 175

Pro Gly Gln Ile Thr Gly Asn Met Ile Cys Val Gly Phe Leu Glu Gly
180 185 190

Gly Lys Asp Ser Cys Gln Gly Asp Ser Gly Gly Pro Val Val Cys Asn
195 200 205

Gly Gln Leu Gln Gly Ile Val Ser Trp Gly Tyr Gly Cys Ala Gln Lys
210 215 220

Asn Lys Pro Gly Val Tyr Thr Lys Val Cys Asn Tyr Val Asn Trp Ile
225 230 235 240

Gln Gln Thr Ile Ala Ala Asn
245

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA probe

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

GCCCCCAACG ATCTTGTCAT CATCATC

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA probe

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

GTTCAGAGTC TTCCTGTCGT ATTGGGG

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA linker

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

GATCCACCAT GAATCCACTC CTGATCCTTA CCTTTGTGGC AG

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 96 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA linker

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

GCACCGGCCG CGGTGGCCTT CCCGACCGAC GATGACGACA AGATCGTCGG CGGGTACACG
TGTGCAGCGA ACTCGATCCC TTACCAGGTC TCGCTG

PATENT CLAIMS

1. A process for the production of trypsin or a derivative thereof in a filamentous fungus, the process comprising

(a) transforming a filamentous fungus host organism with a recombinant DNA vector which comprises a DNA sequence encoding trypsinogen (protrypsin) or a derivative thereof N-terminally fused to a DNA sequence encoding a signal peptide,

(b) culturing the transformed filamentous fungus host organism in a suitable culture medium under conditions conducive to the expression of protrypsin and secretion thereof to the medium, and

(c) recovering the protrypsin or trypsin or derivative thereof from the medium.

2. The process according to claim 1, wherein the filamentous fungus is an Aspergillus sp.

3. The process according to claim 2, wherein the Aspergillus sp. is A. niger or A. oryzae.

4. The process according to claim 3, wherein the DNA vector further comprises a promoter, selected from the group consisting of the A. niger amylase promoters and the A. oryzae TAKA amylase promoter.

5. The process according to any of the claims 1 to 4, wherein said signal sequences is selected from the group comprising the native trypsinogen signal sequences, A. niger amylase signal and the A. oryzae TAKA amylase signal.

6. The process according to any of the claims 1 to 5, wherein said trypsinogen is of animal origin, especially mammalian origin.

7. The process of claim 6, wherein said mammal is a human or a pig.

8. The process according to any of the claims 1 to 5, wherein said trypsinogen is of microbial origin, especially of baterial or fungal origin.

9. A DNA sequence encoding a porcine trypsinogen and having essentially the sequence as given in SEQ ID: 1.

10. A vector comprising a DNA sequence according to claim 9.

11. A host transformed with a vector according to claim 10.

12. The host of claim 11, which is a mammalian host.

13. The host of claim 11, which is a microbial host.

14. The host of claim 13 which is a yeast or fungi.

15. The host of claim 14 which is a filamentous fungi.

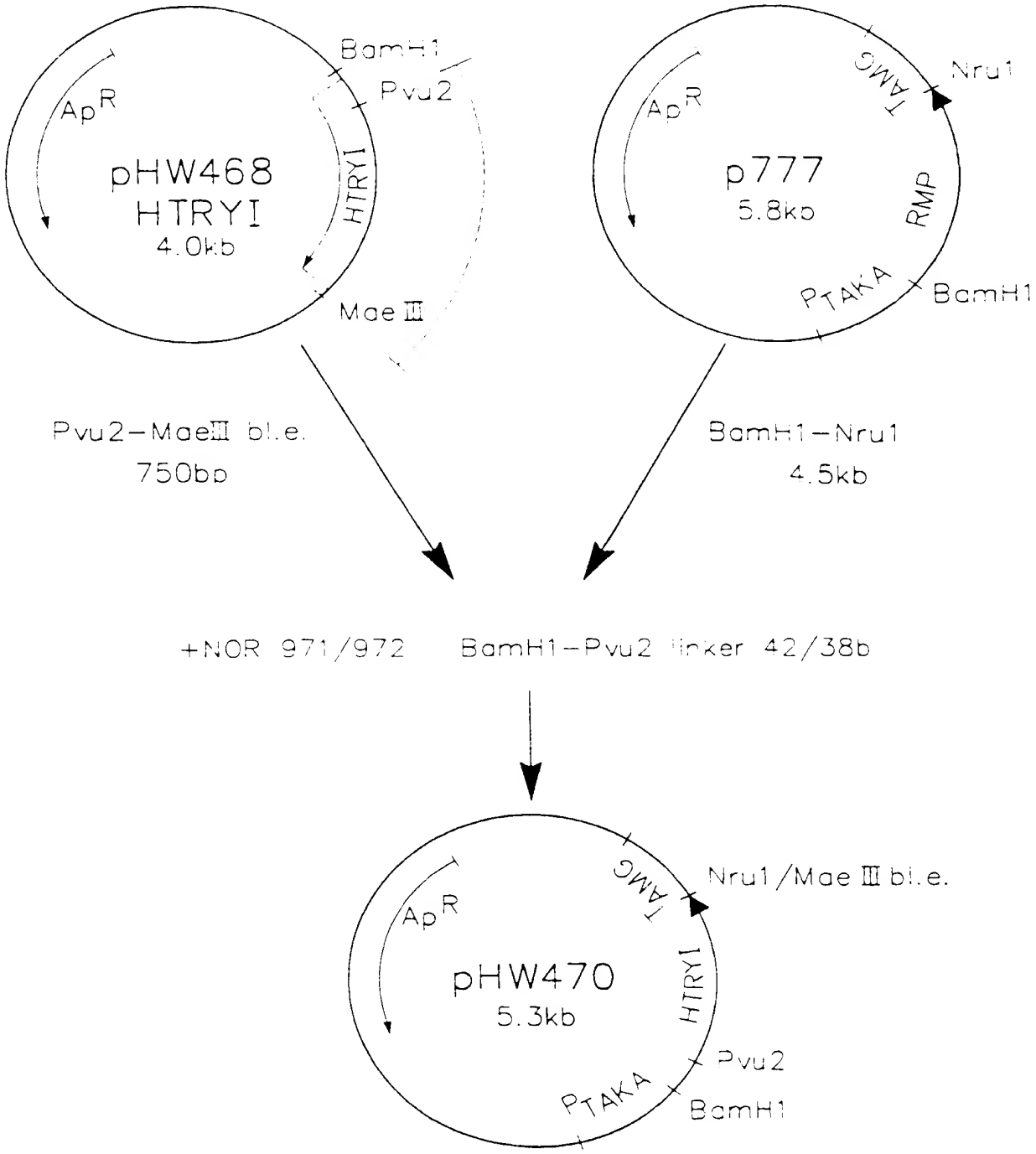
16. A method of recombinantly producing porcine trypsin, the process comprising

(a) transforming a host with a recombinant DNA vector which comprises a DNA sequence encoding porcine trypsinogen or a derivative thereof N-terminally fused to a DNA sequence encoding a signal peptide

that may be the native sequence or another signal sequence or a derivative of such a signal peptide,

(b) culturing the transformed host in a suitable culture medium under conditions conducive to the expression of porcine trypsinogen and secretion thereof to the medium, and

(c) recovering the porcine trypsinogen or trypsin or derivative thereof from the medium.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Nru1
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PTAKA
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4.5kb
+NOR 971/972
BamH1–Pvu2 linker 42/38b
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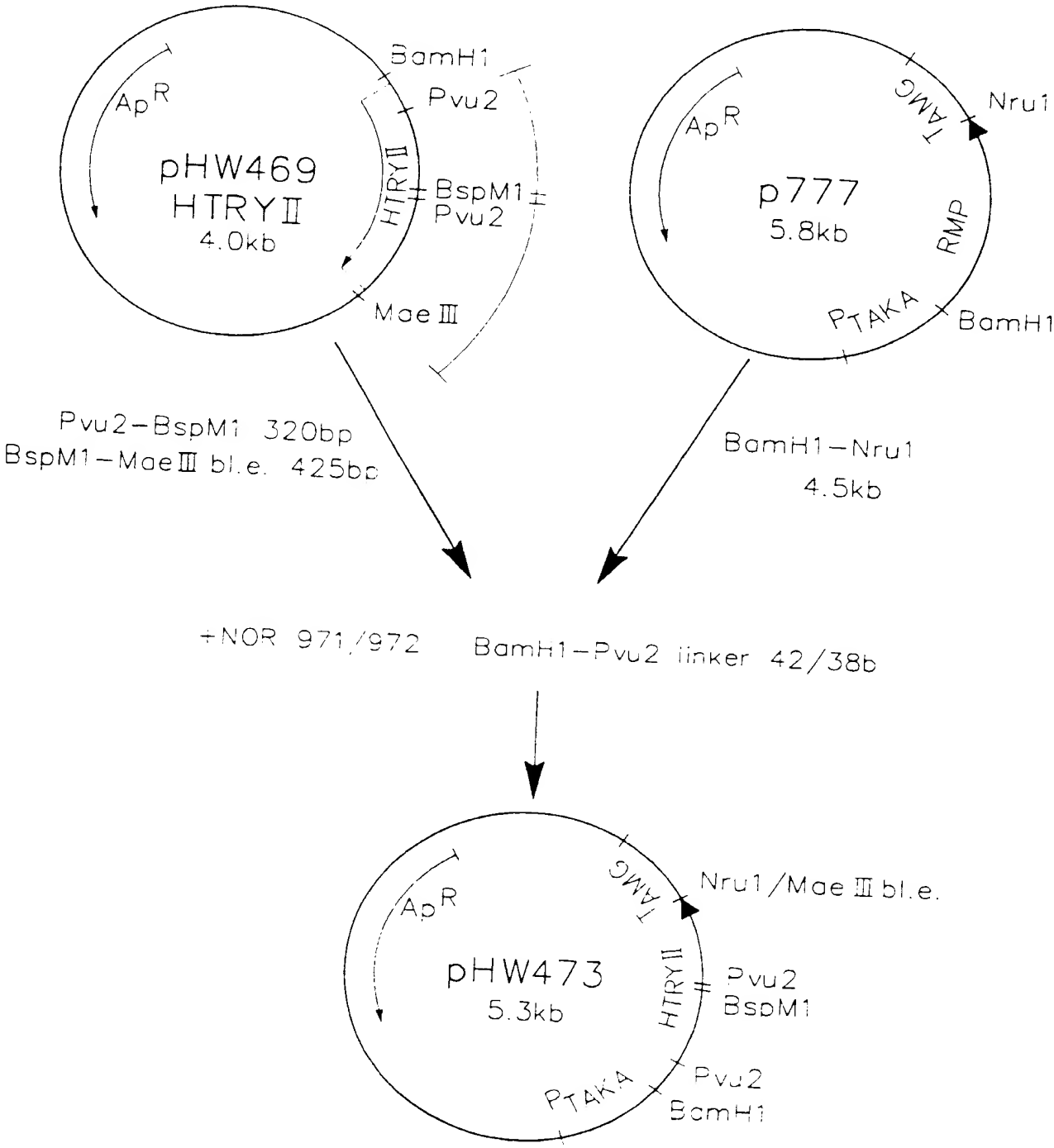
BamH1

Fig. 1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Nru1/Mae III bl.e.
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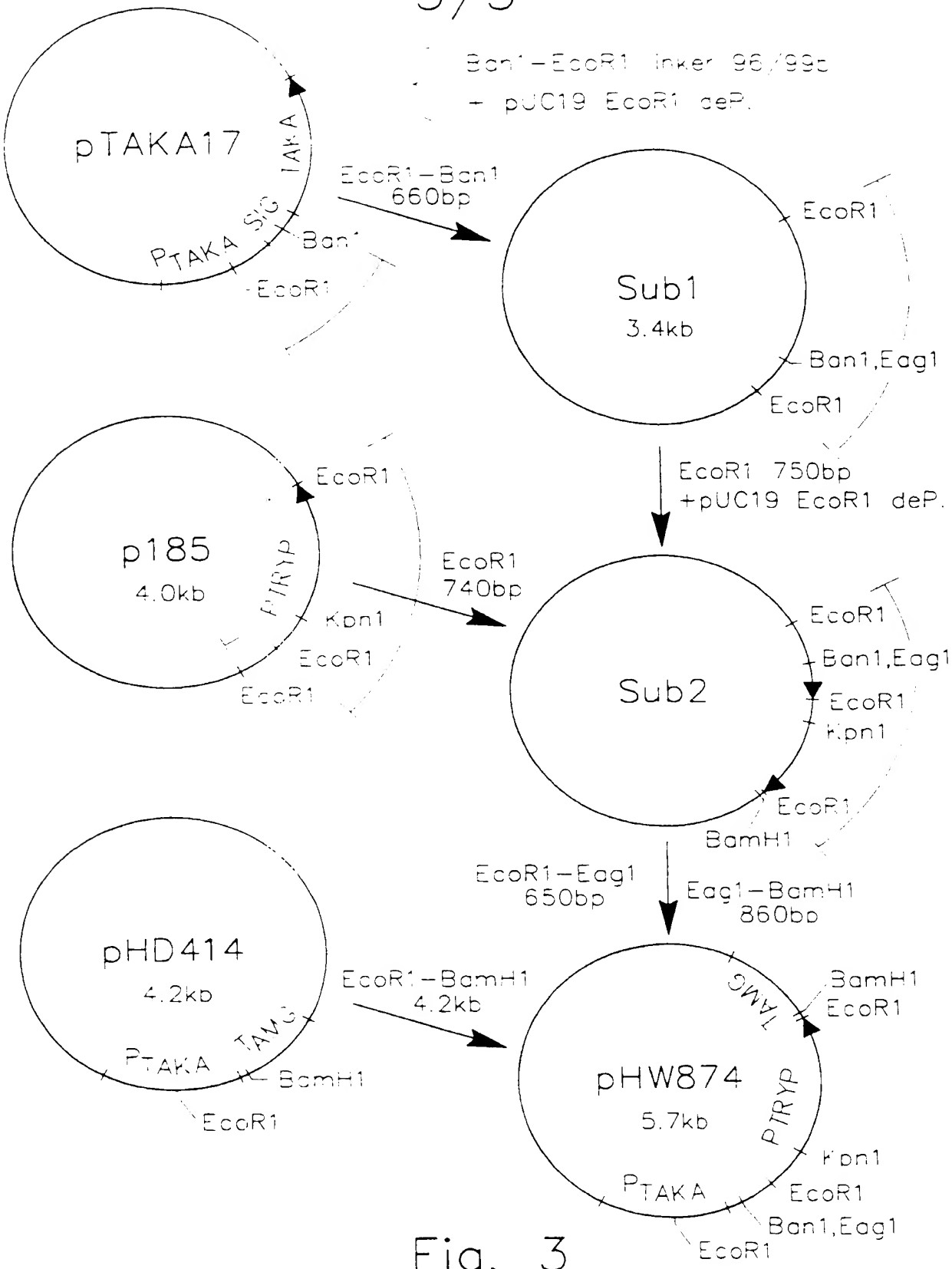
BamH1

Fig. 2

3/3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+ pUC19 EcoR1 deP.
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Sub1
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EcoR1
EcoR1 750bp
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EcoR1
PTRYP
Kpn1
PTAKA
EcoR1
Ban1,Eag1
EcoR1

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00253

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/76, C12N 15/57
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 9425583 A1 (NOVO NORDISK A/S), 10 November 1994 (10.11.94), page 7, line 22 - line 27; page 9, line 21 - line 24	1-8
Y	EP 0597681 A1 (ELI LILLY AND COMPANY), 18 May 1994 (18.05.94), page 2, line 27 - line 29	1-8
Y	Dialog Information Services, file 351, WPI, Dialog accession no. 007590958, WPI accession no. 88-224890/32, SANKYO CO LTD "Human spleen trypsin - used to treat lesions or trauma, without hypersensitive allergic side effects"; & JP,A,63160582, 880704, 8832 (Basic)	1-8

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 October 1996	04 -11- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00253

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9515391 A2 (NOVO NORDISK BIOTECH, INC.), 8 June 1995 (08.06.95), page 1, line 5 - line 11, claim 5	2-5

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00253

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-8

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK 96/00253

According to rule 13.1 an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each define a contribution which each of the inventions makes over prior art.

A search for this "special technical feature" among the independent claims did not reveal such a unifying, novel technical feature. Accordingly the following inventions were found:

1. Claims 1-8 which relate to a process for the production of trypsin. A fungus host is transformed with a DNA sequence encoding trypsinogen.

2. Claims 9-16 which relate to a specific DNA sequence encoding porcine trypsinogen and hosts transformed with this gene. The host is not restricted to fungi.

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/10/96

International application No.
PCT/DK 96/00253

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9425583	10/11/94	AU-A-	6720394	21/11/94
		CN-A-	1125465	26/06/96
EP-A1- 0597681	18/05/94	CA-A-	2102673	14/05/94
		IL-D-	107536	00/00/00
		JP-A-	6225772	16/08/94
WO-A2- 9515391	08/06/95	AU-A-	1101995	19/06/95
		CA-A-	2178007	08/06/95
		EP-A-	0730655	11/09/96
		FI-A-	962288	26/07/96